PB89-224570

Application of a Simple Short-Term Bioassay for the Identification of Genotoxins from Hazardous Wastes

Environmental Health Research and Testing, Inc.
Research Triangle Park, NC

Prepared for:

Health Effects Research Lab., Research Triangle Park, NC
Research Triangle Park, NC

1989

U.S. Department of Commerce
National Technical Information Service
NTIS

TECHNICAL REPORT DATA
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1. REPORT NO. EPA/600/D-89/099	2.
3. RECIPIENT'S ACCESSION NO. PB89-224570	
4. TITLE AND SUBTITLE APPLICATION OF A SIMPLE SHORT-TERM BIOASSAY FOR THE IDENTIFICATION OF GENOTOXINS FROM HAZARDOUS WASTES	5. REPORT DATE
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) S.S. Sandhu,[1] G.N. Acedo[2]	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS [1] U.S. EPA (MD-68), RTP, NC 27711 [2] EHRT, RTP, NC 27709	10. PROGRAM ELEMENT NO.
	11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS Health Effects Research Laboratory Office of Research and Development U.S. Environmental Protection Agency Research Triangle Park, NC 27711	13. TYPE OF REPORT AND PERIOD COVERED ASTM STP 1062, Vol. 2
	14. SPONSORING AGENCY CODE EPA/600/11
15. SUPPLEMENTARY NOTES	

16. ABSTRACT

The proper disposal of hazardous wastes currently generated and clean up of waste disposal sites of the past are challenges facing regulatory agencies in the industrialized nations. The estimation of levels of toxicity is an essential step in prioritizing industrial effluents and solid wastes for treatment and disposal. A number of short-term bioassays has been developed to supplement information from chemical analysis for evaluating the potential of chemical complex mixtures to induce adverse human health effects and environmental contamination. Among these bioassays, plant test systems provide simple, inexpensive, and rapid means of evaluating toxic effects of industrial wastes based on multimedia exposure. Two such assays, Tradescantia paludosa and Zea mays, have been used for monitoring the genotoxic effects of ambient air, municipal wastes, industrial effluents, solid wastes, water sediments, and pesticides. We applied the Arabidopsis embryo assay to evaluate the mutagenicity of complex environmental mixtures including industrial effluents and sludges. The industrial waste samples were tested either unextracted or as dichloromethane aqueous extracts. The significance of short-term plant bioassays for use in environmental assessment will be discussed.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

18. DISTRIBUTION STATEMENT RELEASE TO PUBLIC	19. SECURITY CLASS (This Report) UNCLASSIFIED	21. NO. OF PAGES 12
	20. SECURITY CLASS (This page) UNCLASSIFIED	22. PRICE A03

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

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EPA/600/D-89/099

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APPLICATION OF A SIMPLE SHORT-TERM BIOASSAY FOR THE IDENTIFICATION OF GENOTOXINS FROM HAZARDOUS WASTES

REFERENCE: Sandhu, S. S., Acedo, G. N., "Application of a Simple Short-Term Bioassay for the Identification of Genotoxins from Hazardous Wastes," Symposium on Waste Testing and Quality Assurance: Second Volume, ASTM STP 1062, D. Friedman, Ed., American Society for Testing and Materials, Philadelphia, 1989.

ABSTRACT: The proper disposal of hazardous wastes currently generated and clean up of waste disposal sites of the past are challenges facing regulatory agencies in the industrialized nations. The estimation of levels of toxicity is an essential step in prioritizing industrial effluents and solid wastes for treatment and disposal. A number of short-term bioassays has been developed to supplement information from chemical analysis for evaluating the potential of chemical complex mixtures to induce adverse human health effects and environmental contamination. Among these bioassays, plant test systems provide simple, inexpensive, and rapid means of evaluating toxic effects of industrial wastes based on multimedia exposure. Two such assays, Tradescantia paludosa and Zea mays, have been used for monitoring the genotoxic effects of ambient air, municipal wastes, industrial effluents, solid wastes, water sediments, and pesticides. We applied the Arabidopsis embryo assay to evaluate the mutagenicity of complex environmental mixtures including industrial effluents and sludges. The industrial waste samples were tested either unextracted or as dichloromethane aqueous extracts. These samples produced positive mutagenic results in the Arabidopsis embryo assay.

KEYWORDS: Arabidopsis embryo assay, complex mixtures, genotoxicity evaluations

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INTRODUCTION

Approximately 200 short-term (STT) including both in vitro and in vivo test systems are currently available to evaluate the genotoxic potential of environmental chemicals. Most of these bioassays were developed in the early 70's after a discovery by Dr. Bruce Ames [1] of the University of California, Berkeley, CA, that many of the carcinogens can be identified by using simple microbial mutagenicity bioassays. These STT employ either in vitro procaryotic and eucaryotic cells or in vivo animal test systems. Substantial progress has been made in utilizing these assays for identifying mutagens and carcinogens in a variety of products such as agricultural chemicals, drugs, consumer products, industrial chemicals, etc. Since humans are rarely exposed to individual chemicals, but exposure occurs mostly to a mixture of chemicals, the STT technology has been applied for evaluating the genotoxicity of complex chemical mixtures such as industrial emissions, effluents, and hazardous waste samples, and for evaluating the level of mutagenicity in the ambient environment [2].

One of the STT that appears very useful for identifying mutagens and presumptive carcinogens in the environment is the Arabidopsis embryo assay (AEA). Originally developed by Andreas Muller [3,4], this assay has been used to assess the mutagenicity of over 120 chemicals [5,6,7,8] and complex mixtures [9,10]. One of the easily detectable genetic endpoints in the AEA is the loss of chlorophyll pigmentation in the developing embryos of the mutant population. As an in vivo bioassay, Arabidopsis offers certain unique advantages which include: (1) short study period (4 weeks), (2) economy of space (200 plants can be grown in a petri dish), (3) high fecundity, (4) easy to grow and score for mutation, (5) multiple genetic endpoints, and (6) induced mutations are observed in the progeny of the exposed population. Although data are limited, it appears that this small crucifer has the capability to biotransform promutagens to mutagenic levels [6].

Since the usefulness of AEA for evaluating the mutagenicity of individual compounds has been established, we have attempted to extend its utility for assessing the genotoxic hazard of complex environmental mixtures. In the study reported here we have applied AEA for analysis of the genotoxicity of five hazardous waste samples obtained from a phenyl mercuric acetate plant.

MATERIALS AND METHODS

Hazardous Waste Samples

Two liquid effluent and three solid waste samples from a mercuric acetate plant were received through Versar, Inc., New Jersey. The liquid samples were Cosan Scrubber water and Cosan Separated water. The Cosan Scrubber water sample was very alkaline (pH 11.0), whereas the Cosan Separated water sample was extremely acidic. The three solid samples were identified as Noudex Presscake No. 2 (NP2), Noudex Presscake No. 3 (NP3), and Noudex-Hg (N-Hg) recovery sludge. These

samples were evaluated for their genotoxic effects either as crude samples or after extraction with methylene chloride or water.

Sample Preparation

The pH of the liquid samples was adjusted to 7.0 for Cosan Scrubber and to 5.0 for Cosan Separated to allow the growth of Arabidopsis. Concentrations of the sample ranging from 0.1-1,000 mg/ml were made with buffered mineral medium [11]. A total volume of 1 ml of the test solution contained in scintillation vials was used for treatment.

The solid samples were tested in their crude form (as received) and as aqueous or methylene chloride extracts. For testing the crude samples, seeds were exposed by planting them directly in mixtures containing various proportions of the test material and Promix™ medium (Premier Brands, New Rochelle, NY 10804). The mixtures were prepared by kneading the desired amount of Promix™ and the sample together in a plastic bag and putting the mixture in petri dishes where the Arabidopsis is grown.

Aqueous extracts of the solid samples were obtained by preparing 1:1 (v/v) mixtures of sample and deionized water. These mixtures were allowed to stand for 1 h, centrifuged at 4,500 rpm for 10 min, and the supernatants were collected. Dilutions of these extracts were tested for evaluating the genotoxicity of these samples.

Methylene chloride extracts were solvent exchanged to DMSO to a concentration equivalent to 1 mg/ml, and further dilutions ranging from 0.01-0.50 mg/ml were made and evaluated for their genotoxicity.

Arabidopsis Assay Protocol

For testing liquid samples and sample extracts, approximately 300 seeds of Arabidopsis thaliana (Columbia wild type), contained in a small cloth bag, were immersed in each vial of test solution for about 12-15 h at room temperature. At the end of the treatment period, the seed bags were rinsed under running tap water for 1 h. The washed seeds were dispersed in water and planted in glass Petri dishes (85x75 mm) containing pasteurized Promix™ medium. The dishes were subsequently placed in a growth chamber set to provide a 16-h photoperiod and a temperature of 24° C.

Crude samples (nonextracted solid samples) were tested by planting the Arabidopsis seeds in Petri dishes containing sample/Promix™ mixtures, then placing the dishes in the growth chamber. The plants were watered daily with Hoagland nutrient solution.

After 3 weeks of growth, when the seeds were mature but not yet brown, three fruits from each of the 100 plants selected at random for each concentration were opened with sharp forceps. The embryos within these fruits were scored for white (mutant) embryos among the normal green embryos (Fig. 1). The extent of sterility induced by each concentration was also determined. Sterility is manifested as

FIG 1. -- A Arabidopsis thaliana plant with segregating white and green embryos.

empty spaces within a fruit or, in severe cases, no fruits are formed at all along the stem.

Each experiment contained a concurrent positive control (0.2% ethyl methanesulfonate) and a solvent negative control. The data presented are an average of the two experiments except for the methylene chloride extracts for which the results of a single experiment are given. Confidence intervals (CI) were determined using the statistical methods of Ehrenberg [12].

In evaluating the experimental data, a sample was considered mutagenic to Arabidopsis if it induced a twofold or greater increase in the number of chlorophyll mutants at two or more doses as compared to solvent control or if it induced dose-related increases at three or more doses.

RESULTS

The data on the mutagenicity of the liquid samples, Cosan Scrubber water and Cosan Separated water, presented in Table 1, indicate that both samples are mutagenic to Arabidopsis. A slight dose response in mutagenicity is indicated by Cosan Scrubber water, and although Cosan Separated water shows a similar trend, there was no further increase in mutagenicity beyond the concentration of 1 mg/ml (Fig. 2), which may be due to cytotoxic effects. In addition to mutagenicity, a high degree of sterility was induced by 10 mg/ml of Cosan Scrubber water and the undiluted (1,000 mg/ml) Cosan Separated water (Table 1). Germination of the treated seeds was considerably reduced at higher concentrations. These results must, however, be viewed with caution since the pH of both samples had to be drastically adjusted prior to testing to allow growth of Arabidopsis. The alkaline Cosan Scrubber sample was adjusted using whole pellets of KOH. Although these pH changes were necessary to allow testing, it is quite probable that some components of these complex mixtures were substantially altered in the process. The nature of these alterations and their effect on the overall mutagenicity of the samples is not known.

TABLE 1 -- Mutagenicity of Cosan Liquid samples in the Arabidopsis embryo assay (data are an average of two experiments).

Sample	Concentration (mg/ml)	Germination (%)	% of Fruits Containing Mutants + CI	Sterility (%)
Cosan Scrubber Water	0.1	95.5	15.5 ± 4.94	1.5
	1.0	97.5	19.5 ± 8.90	8.5
	10.0	99.0	20.8 ± 13.77	14.0
	100.0	73.0	23.6 ± 7.75	2.0
	1000.0	50.0	32.0 ± 4.76	3.5
Cosan Separated Water	0.1	97.5	18.0 ± 1.96	4.5
	1.0	89.0	22.6 ± 8.64	7.8
	10.0	96.0	24.4 ± 13.95	4.6
	100.0	55.0	28.9 ± 16.60	2.0
	1000.0	52.0	28.6 ± 9.29	40.0
Solvent Control (mineral medium)	0	100.0	4.0 ± 2.78	0
Positive Control (EMS)	0.20	97.0	80.0 ± 1.96	64.0

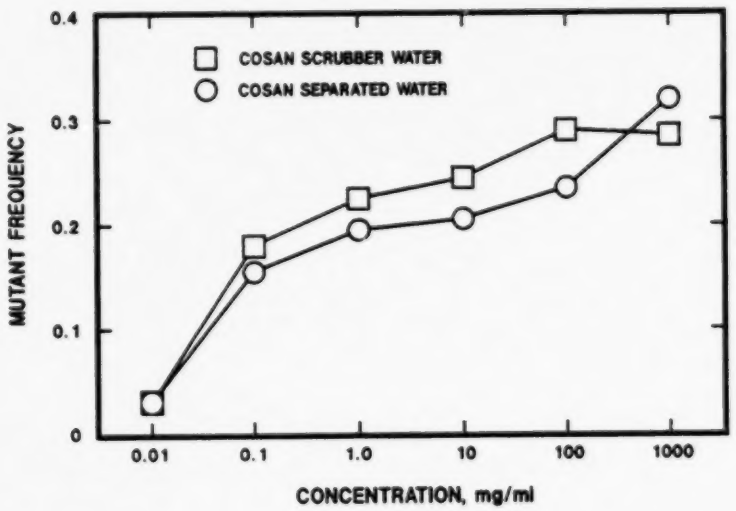

FIG 2. -- Mutagenicity induced in Arabidopsis by the Cosan liquid samples.

The solid waste samples, NP2, NP3, and N-Hg recovery sludge were tested as unextracted crude samples as well as after extraction with methylene chloride and water. The toxicity range-finding experiments

showed that the unextracted crude samples were very toxic for Arabidopsis. Therefore, these materials were tested for mutagenicity after various dilutions with Promix™ medium. The mutagenicity of these samples for Arabidopsis is shown in Table 2. NP2 was very toxic to Arabidopsis. Even after mixing 1 part of the sample with 9,000 parts of Promix™ the survival of the treated plants was only 50%. At this low concentration, NP2, however, induced about four times more chlorophyll mutations than the solvent control (Table 2). It was interesting to note the relatively low degree of sterility (7.7%) induced in the surviving plants, indicating that the observed toxicity may be primarily due to inhibition of growth rather than interference with genetic mechanisms. Proportions of sample lower than 1:9,000 were not tested due to difficulties encountered in accurately measuring and mixing with Promix such a small amount of the sample.

TABLE 2 -- Mutagenicity of three solid waste samples in Arabidopsis (data are an average of two experiments).

Sample	Proportion (Sample: Promix™ v/v)	Germination (%)	% of Fruits Containing Chlorophyll Mutants + CI	Degree of Sterility (%)
Noudex Presscake No. 2	1:9000	50	19.2 ± 5.50	7.7
Noudex Presscake No. 3	1:4000	85	15.0 ± 5.90	8.3
	1:2000	95	19.3 ± 2.70	13.8
	1:1000	94	20.5 ± 8.44	17.9
Noudex Hg	1:4000	80	19.0 ± 7.86	6.5
	1:2000	83	16.5 ± 4.91	8.5
Promix™ only		98	5.0 ± 1.96	3.0

NP3 and N-Hg recovery sludge were relatively less toxic to Arabidopsis as compared to NP2. Nevertheless, both samples were mutagenic for Arabidopsis (Table 2).

The results of the treatment with methylene chloride-extracted samples are shown in Table 3. All three samples were positive for mutagenicity; however, dose-related response was not observed for these extracts. For lack of sample availability, testing at doses lower than 0.1 mg/ml and a repeat experiment could not be performed.

Table 4 shows the mutagenicity for Arabidopsis after treatment with the aqueous extracts from each of the three solid samples. NP3 and N-Hg recovery sludge samples showed dose-related mutagenic response although the mutagenicity values were much lower than that of NP2. A high degree of sterility was induced by N-Hg especially at 100% concentration of the extract.

TABLE 3 -- Mutagenicity of methylene chloride extracts in Arabidopsis (data are from one experiment).

Sample	Concentration (mg/ml)	Germination (%)	% of Fruits Containing Chlorophyll Mutants	Degree of Sterility (%)
Noudex Presscake	0.01	90.0	25.6	15.0
No. 2	0.10	91.0	22.0	4.0
0.25	95.0	22.6	1.0	
0.50	88.0	22.0	13.0	
Noudex Presscake	0.01	98.0	12.0	10.0
No. 3	0.10	86.4	13.0	12.0
0.25	85.0	18.0	0	
0.50	82.7	13.0	12.0	
Noudex Hg	0.01	100.0	8.6	1.0
0.10	100.0	14.0	10.0	
0.25	89.0	18.0	3.0	
0.50	87.3	14.0	13.0	
Solvent Control (DMSO)	100.00	86.5	7.0	7.0

TABLE 4 -- Mutagenicity of aqueous extracts in Arabidopsis.

Sample	Concentration (%)	Germination (%)	% of Fruits Containing Chlorophyll Mutants + CI	Degree of Sterility (%)
Noudex Presscake	1.0	85.0	18.9 ± 5.70	0
No. 2	10.0	67.0	26.5 ± 0.98	1.0
	100.0	50.0	21.5 ± 2.95	9.0
Noudex Presscake	1.0	100.0	5.0 ± 3.93	0
No. 3	10.0	100.0	8.0 ± 5.90	0
	100.0	65.0	13.7 ± 6.55	2.8
Noudex Hg	1.0	100.0	6.0 ± 3.93	9.0
	10.0	70.0	8.3 ± 7.27	6.0
	100.0	63.0	11.5 ± 6.88	18.0
Solvent Control (water)	0	100.0	5.0 ± 1.96	0
Mutagen Control (EMS)	0.20	100.0	81.5 ± 0.88	80.0

DISCUSSION

The identification and proper disposal of hazardous wastes are challenges facing regulatory agencies in the industrialized nations. At present, most of the regulatory agencies, including the U.S. EPA, use chemical components of the industrial wastes as a basis for hazard evaluation [13]. However, chemical analysis is currently limited to the detection of only a few known toxic compounds, whereas numerous other potentially harmful genotoxins may be undetected. Even if all the chemicals present in hazardous wastes were identified (which is an improbable task), chemical analysis alone will not provide information on the synergistic and antagonistic effect that may result from the interaction of these complex chemical mixtures. Therefore a biological evaluation along with their chemical analysis has been repeatedly emphasized for assessing the health hazards of complex chemical mixtures [2,9].

A large assortment of STT may be utilized for the evaluation of hazardous industrial wastes, and plant bioassays are uniquely suitable for this purpose. Certain bioassays such as Zea mays [14], Tradescantia micronucleus assay [15], and Tradescantia gene mutation assay [16] have been very useful for evaluating the levels of genotoxins in the terrestrial and aquatic environment [17]. The primary advantages of the plant systems are their simplicity, cost effectiveness, and utility in assessment of simultaneous multimedia exposure. In contrast to cell culture bioassay, one does not have to worry about microbial contamination.

The purpose of analyzing the hazardous waste is twofold: (1) to assess its overall health hazards for its proper disposal and (2) to identify the specific components of the waste responsible for the particular effects so that control technology can be applied to minimize the production of toxic chemicals. In the study reported here, we have applied a simple bioassay in an attempt to accomplish the first objective. Our choice of test samples, obtained from a phenyl mercuric acetate plant, was based on their availability. The data presented in this study are based only on results from crude samples and on methylene chloride and aqueous extracts. Although extraction reduced cellular toxicity as shown by seed germination, it did not alter the qualitative mutagenicity results of the samples.

ACKNOWLEDGMENT

Although the research described in this article has been supported by the United States Environmental Protection Agency through on-site support service contract 68-02-4456 to Environmental Health, Research and Testing, Inc., it has not been subjected to Agency review and therefore does not necessarily reflect the views of the Agency and no official endorsement should be inferred. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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